CD105/ENDOGLIN EXPRESSION IN A MOUSE MODEL OF ACUTE MUSCLE CONTUSION

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ABSTRACT

Objectives. Muscle contusion is the most common form of muscle injury and the muscle endogenous regenerative capacity can compensate for non-extensive damage. However, severe traumatic injuries may overcome this capacity. More effective therapeutic strategies are still needed, so the exploration of the molecular context during muscle regeneration might provide new insights.

Materials and methods. We investigated the expression pattern of a reputed angiogenic molecule, CD105/endoglin, by measuring the tissue and serum concentration by multiplex assay in normal and *Dmd*^{mdx} dystrophic mice by following the distribution of the cellular sources by immunofluorescence in a mouse model of acute muscle contusion.

Results. Maximal tissue concentration in normal animals was obtained 48h post-injury, and then started to decline, with only one other significantly increased value 5 days after injury. In dystrophic mice, tissue levels were globally much higher than in normal animals and started rising 1h post-injury and were maintained elevated all along the regeneration process. In situ immunolabelling highlighted the increased positive population mostly in the inflammatory areas. Double staining separates distinct subsets based on hematopoietic marker CD45 and the endothelial marker CD31 co-expression in endoglin positive cells.

Conclusions. This study offers a timeline of endoglin expression during normal and pathologic muscle regeneration, providing evidence that the major wave corresponds to the inflammatory stage of muscle regeneration, deriving from multiple cellular sources such as endothelial cells, blood cells and also other interstitial cells that become activated during this process.

Keywords: angiogenesis, CD105, endoglin, muscle regeneration

INTRODUCTION

Mechanical injuries are the most common type of the skeletal muscle injuries. Acute skeletal muscle damage determinates fiber disruption, oxidative stress and inflammation. The main characteristic of skeletal muscle is the capacity to regenerate after injury and dependents upon numerous factors including inflammatory response, growth factor synthesis and subsequent activation of endogenous progenitor cells for muscle and blood vessels reconstruction (1,2).

One of the molecules strongly expressed by proliferating vascular endothelial cells (ECs) is endog-

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lin/CD10, an auxiliary homodimeric transmembrane glycoprotein receptor for TGFβ1and 3 (3,4) and modulates TGF-β – dependent cellular responses such as cellular proliferation, differentiation, extracellular matrix synthesis and accumulation, angiogenesis, and vascular integrity (5-7). However, different recent studies support the idea that endoglin participate in a signalling pathway that is critical for ECs responses to TGF-beta family members (8,9).

Endoglin is expressed not only in ECs but also in different other cell types such as normal smooth muscle cells, stromal cells of mesenchymal origin (10), mesenchymal and hematopoietic stem cells (7), activated monocytes but not peripheral blood monocytes and macrophages (11). Also, fibroblasts from cardiac muscle express endoglin and modulates profibrotic effects of angiotensin II (8).

Persistent exposure to TGF-β superfamily cytokines play an important role in pathogenesis of tissue fibrosis in variety of disorders including regenerating muscle and muscular dystrophy (12) and the expression of endoglin by ECs was found greatly increased after ischemia and reperfusion in the kidney, hind limbs, and heart during reactive hypoxic angiogenesis and also in inflamed tissues and healing wounds (5,13).

This study was designed to evaluate the expression of the TGF-β1 receptor endoglin, in a mouse model of muscle crushing injury during tissue regeneration in both normal and pathological conditions such as muscular dystrophy.

MATERIALS AND METHODS

Muscle contusion model

Animal models of muscle contusion were optimized on 2.5 months old CD1 and *Dmd*^{mdx} dystrophic mice. The experiments were carried out in accordance with international guidelines for animal experiments and were approved by the Committee on the Ethics of Animal Experiments of „Victor Babes“ Institute of Pathology.

Left leg of each animal was crushed for 2 min with a forceps, 1 cm away from the distal joint. Blood and gastrocnemius muscle of 3 different animals was harvested ateach time-points starting 1 hr. post-injury and then during the 2nd, 3rd, 5th, 7th, 14th and 21st day. The contralateral, gastrocnemius muscle and muscles harvested from 7 non-injured animals were used as controls. The samples were frozen in liquid nitrogen and further used for sectioning or for homogenization for xMAP assay

Multiplex assay

The xMAP assay was performed on a Luminex® 200™ platform (Luminexcorp, Austin, TX, USA) according to the manufacturers' protocols. The plates were read using Luminex 200 system. Data acquisition and analysis were performed using xPONENT 3.1 software. Each sample was run in duplicate.

In situ immunofluorescence

Sections were fixed and incubated overnight, at 4°C, with the primary antibodies endoglin (EDM Millipore, 05-1424, 1:100), laminin (Sigma-Aldrich, St. Louis, MO, USA, L9393, 1:150), CD45 (Abcam, Cambridge, UK, ab25386, 1:150) and CD31 (Novus Biologicals, Littleton, USA , NB100-164 , 1:100). After washing with PBS, the appropriate AlexaFluor 488 or 546 secondary antibodies (Molecular Probes, Life Technologies, Carlsbad, CA, USA) were applied for 1 hr. at room temperature. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma), 1.0 μg/mL. Negative controls were obtained by the same protocol, but omitting the primary antibody. Sections were analysed with Nikon TE300 microscope equipped with a Nikon DS-Qi1 camera and NIS Elements software (Nikon Instruments, Melville, NY, USA) with Nikon PlanApo 20x and 40x objectives, and the appropriate fluorescence filters. The figures were assembled using Adobe Photoshop CS3.

STATISTICS

Results are expressed as mean ± standard error of the mean of three independent experiments. The statistical analysis was performed using SPSS-17 statistical software (IBM, NY, USA). Differences were considered significant when p values $*p<0.05$.

RESULTS

Endoglin concentrations determined by xMAP assay showed significant increases in injured tissue samples versus normal, non-injured controls after 48 hrs after the infliction of injury, when we recorded the maximum levels, with a 1.9 fold increase. After that, endoglin concentration started to decrease, with only one other significant level versus normal control at 5 days post-injury (Fig. 1 a,c).

When compared to corresponding contralateral tissue samples, we found significantly increased levels from 48 hrs and up to 7 days post-injury (Fig. 1 a).

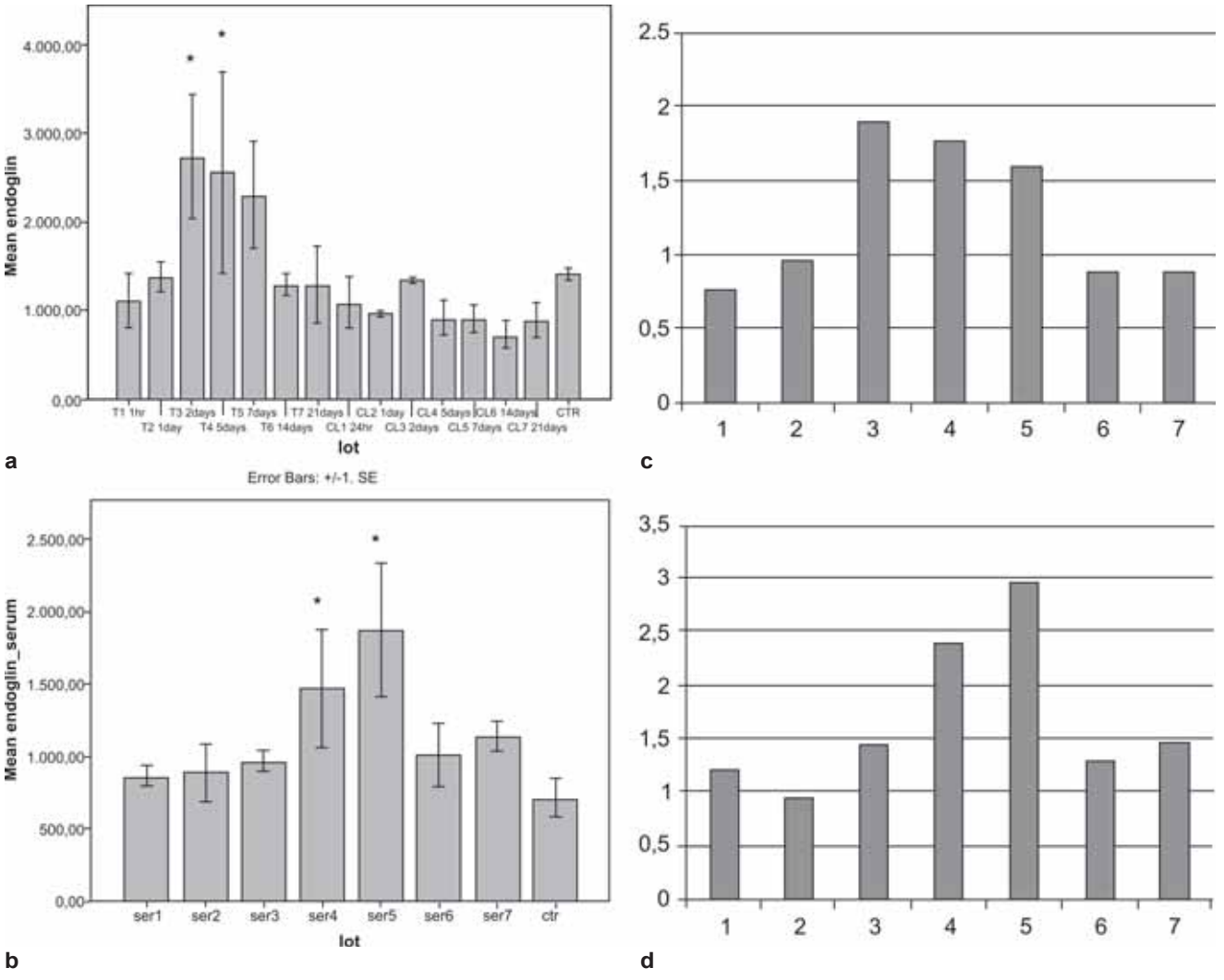

FIGURE 1. *Normalized endoglin concentrations at specified time-points during muscle regeneration. (a) in tissue and (b) serum samples (c) average of fold modification versus average non-injured controls for endoglin concentration in tissue lysate and (d) serum. Data represent the mean and SEM of three independent experiments, each performed in duplicate; *p < 0.05*

Moreover, significant increases versus normal, non-injured controls were measured in serum samples after 5 and 7 days post-injury when we registered the maximum 2.94 fold increase (Fig. 1 b, d).

In *Dmd*^{mdx} dystrophic mice, endoglin levels in injured tissue lysate started rising 1 hr after the injury and were maintained elevated up to 21 days, with 2.91 fold increase at 5 days (Fig. 2 a). Tissue levels were much higher when compared with non-injured controls from normal mice (Fig. 2 b).

In situ immunolabelling experiments proved that endoglin is expressed by vascular cells such as smooth muscle and endothelial cells even in normal tissue samples (Fig. 3 a), but the number of endoglin positive cells gradually increases during muscle regeneration up to 7 days post-injury in all connective tissue compartments based on an influx of mononuclear cells, not surrounded by a basal lamina, as demonstrated by double staining with laminin, a prominent component of basal laminae (Fig. 3 b-d). This population started to fade toward the end of the second week after the injury (Fig. 3 e).

Most cells co-express the endothelial cell marker CD31 and belong to the endothelium of newly formed blood vessels (Fig. 3 f) and activated endothelial cells located in their close proximity (Fig. 3 g, arrows) or in the injured endomisium (Fig. 3 h and i). The arrow in panels h and i indicate the same endothelial cells in the last phase of mitotic division, that co-expresses endoglin. However, there are many other mononuclear cells that do not co-express CD31.

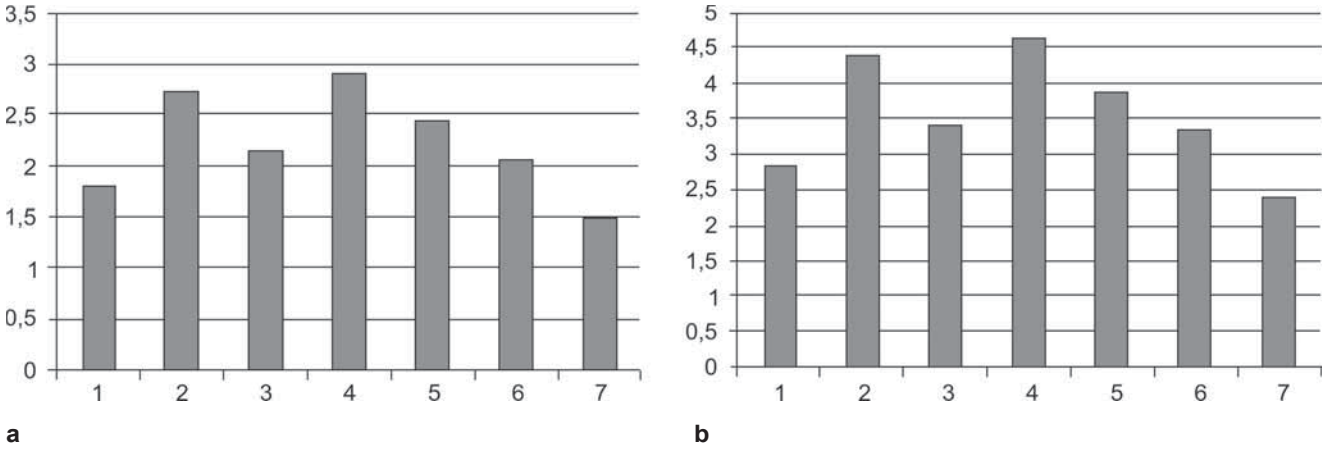

FIGURE 2. *Average endoglin concentration fold modification in tissue lysate from injured dystrophic mice versus average non-injured controls dystrophic mice (a) and normal mice (b)*
In Dmd^{mdx} dystrophic mice, endoglin levels in injured tissue lysate started rising 1 hr after the injury and were maintained elevated up to 21 days, with 2.91 fold increase at 5 days (Fig. 2 a). Tissue levels were much higher when compared with non-injured controls from normal mice (Fig. 2 b).

Double immunofluorescence experiments for endoglin and hematopoietic marker CD45 proved that during muscle regeneration there is an important population of endoglin positive cells not expressing CD45 (Fig. 3 j and k), especially in the beginning of the regeneration process (Fig. 3 j, arrow heads).

DISCUSSION

An immediate response to muscle injury is the local activation of the innate immune response. One of the earliest events is the invasion of the damaged site by inflammatory cells, particularly monocytes that secrete pro- and then anti-inflammatory cytokines. By TGFβ expression, macrophages play an important role in new blood vessels formation (14). Angiogenesis is a key process during tissue regeneration. It involves the formation of new vessels in two phases: activation and maturation. The whole process represents a complex interaction of various trophic molecules amongst which TGF-β and their specific receptors play an important role.

Most research studies revealed that the cellular and tissue distribution of endoglin is an important factor for its angiogenic role: control of cell proliferation, migration, and capillary tube formation (15). Our data demonstrated that endoglin expressing cells are usually endothelial cells, by CD31 co-expression, but also other types of interstitial cells distributed around pre-existing and newly formed blood vessels. Such cells are not surrounded by a basal lamina like pericytes and smooth muscle cells and some of them do not express hematopoietic marker CD45, so not all of them are blood derived. Recent studies consider endoglin as a more specific marker for new, immature blood vessels (16) that do not yet express CD31 and CD34.

However, there is an important population of blood-derived endoglin positive cells that gradually increases toward 48 hrs post-injury and then slowly declines toward the second week after trauma. Consistent with our results, previous studies showed that activated mononuclear cells express high levels of endoglin during monocyte-macrophage transition (17) which corresponds with the early stage of inflammation during skeletal muscle regeneration when we measured the maximum levels during regeneration in normal muscle.

We found that mdx mice show a different expression pattern for endoglin expression along regeneration process, with higher basal levels and much higher levels at all selected time-points, starting to increase much earlier, only 1hr after the injury. Increased levels versus non-injured controls were maintained up to 21 days, peaking 2 days later then in normal injured tissue.

In dystrophic mdx mice muscles undergo frequent cycles of necrosis and regeneration, with subsequent progressive degeneration of the muscle (18). They show common pathologic features, such as altered Ca^{2+} homeostasis, infiltration of muscle tissue by inflammatory immune cells, increased levels of proinflammatory and profibrotic cytokines, and intracellular accumulation of reactive oxygen species (ROS) production even in the absence of injury (19). Immune cells infiltration precedes the onset of muscle dystrophy and persist

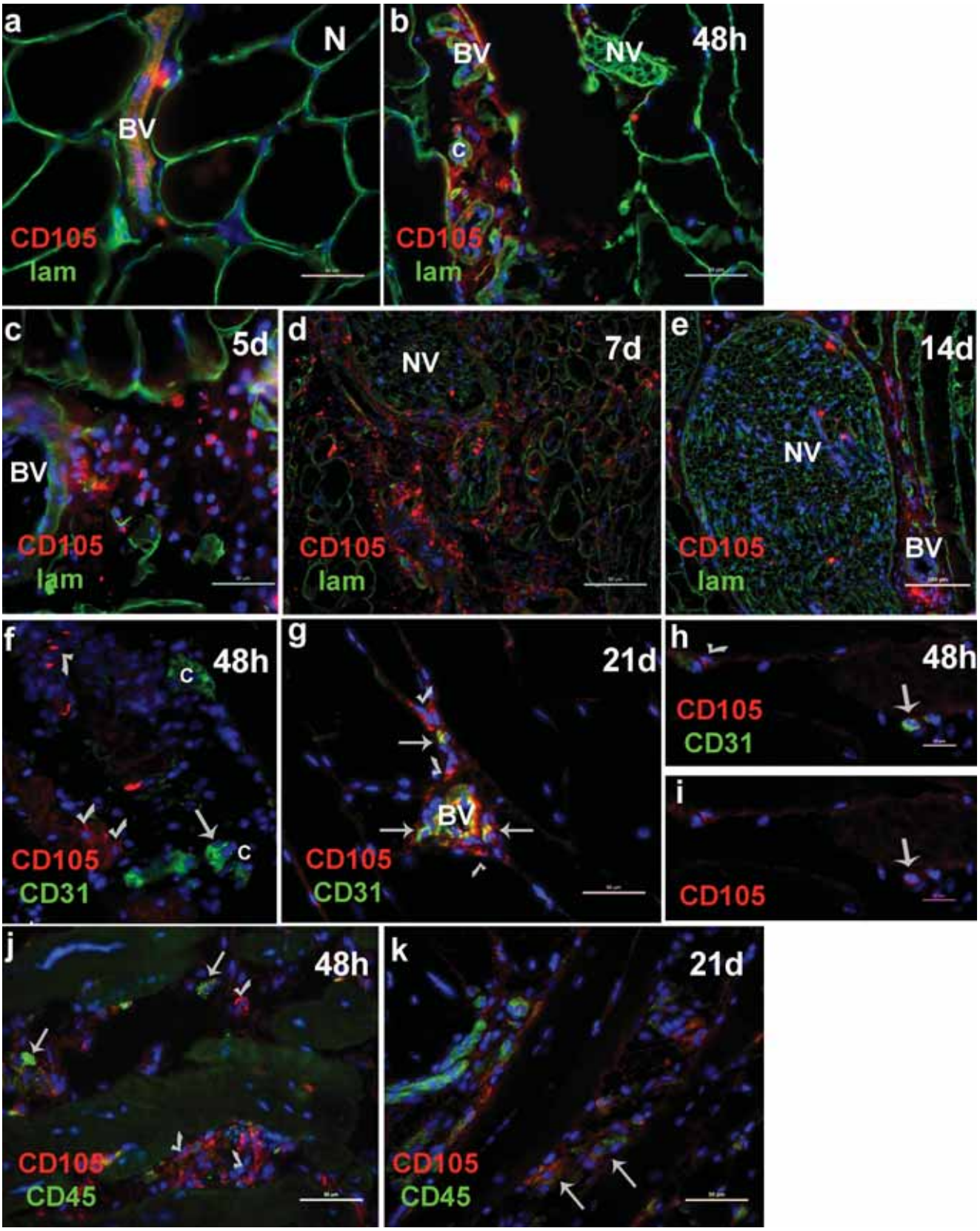

FIGURE 3. *Immunofluorescent labelling shows the distribution of endoglin positive cells (red) in normal control (a) and in interstitial compartments of injured samples at specified time-points during regeneration process (b, c, d, e). Muscle cells were outlined by laminin labelling (green). Double immunostaining for endoglin (red) and endothelial marker CD31 (green) (f, h, i) and hematopoietic marker CD45 (green) (j, k). highlights double positive cells (arrows) together with endoglin positive cells (arrow heads). Original magnification, 400x except panels d and e(200x).*

also in late stages being considered as a specific response that contributes to inflammation and muscle injury formation and progression. Fibrosis is a prominent pathological feature in muscular dystrophy (20). Research studies revealed that the TGF-β1 levels are increased in muscle tissues in mdx animal models and endoglin modulates TGF-β central role in stimulating fibrosis (19).

TGF-β is produced by many cell types in dystrophic muscles (myofibers, fibroblasts, and infiltrating immune cells) and can act as a mediator to increase synthesis and accumulation of matrix proteins (21) having a great contribution to endomysial fibrosis (19). Furthermore, increased levels of TGF-β1 inhibit satellite cell activation and impair myocyte differentiation (12) so it would be expected for its receptor expression, endoglin, to decline during the second week post-injury during normal muscle regeneration, and to remain elevated in the case of dystrophic muscle, as demonstrated by our results.

Increased soluble endoglin levels were found to be related to endothelial damage and dysfunction (22) which elucidate the 48 h peak in our model of acute muscle contusion.

Soluble forms are generated by the cleavage of the extracellular domain of endoglin by membrane-type metalloprotease-14 (MMP-14) and serve as a natural antagonist for TGF-β signalling. It has binding sites for various members of the TGF-β superfamily interfering with their binding to the functional receptors, inhibiting angiogenesis (15) and may contribute to elevated vascular resistance (10). Consistent with this data, in our experimental model the significant increase in endoglin serum levels at 5 days post-injury corresponds to the beginning of tissue endoglin level decline. This could be explained as the most prominent cellular source is represented by newly formed blood vessels and the angiogenic process starts to decline toward the end of the first week after the injury.

CONCLUSIONS

We provide a time-line for endoglin expression as a marker of neo-angiogenesis during muscle regeneration after acute mechanical trauma in both normal and pathologic conditions provided by mdx, dystrophic mice. The most important cellular source is represented by inflammatory cells offering higher levels during inflammatory stages which further activate angiogenesis with additional endoglin sources from newly activated endothelial cells. In mdx mice, the overall tissue concentration is much higher due to constant inflammation, higher levels being registered later then in normal mice suggesting a late onset for the angiogenic process.

Acknowledgments

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